Short review

Seed biologists beware: Estimates of initial viability based on ungerminated seeds at the end of an experiment may be error-prone

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Running title: Seed biologists beware

Abstract: Seed viability is routinely measured on seeds that fail to germinate at the end of an experiment. Together with the number of germinants, this is used to estimate viability of the seeds at start of the experiment (i.e., initial viability) and provides the comparative basis on which germination success is determined. Perusal of the literature shows that sometimes (perhaps often, as the problem has yet to be recognized or reported) prolonged duration in the treatment, especially the control where little germination occurs, can lead to loss of viability. This results in underestimation of initial viability if that treatment is used. I caution against the routine use of end-of-trial germination and viability of ungerminated seeds as an estimate of initial viability in determining germination success of various treatments. I explore ways to deal with the problem but the preference is for estimates of initial viability to be undertaken on a separate sample of seeds concurrently with the experiment as this avoids the risk of seed death during the trial.

Keywords: experimental conditions; germination; seed viability estimation; seed viability loss

The problem

Determining initial viability is a vital part of testing the germination potential and requirements of seeds, since germination success can only be compared against seeds that were viable and therefore capable of germinating. Researchers usually estimate seed viability at the start of a trial/experiment (initial viability) on the condition of seeds remaining at the end of the trial plus those that had germinated. A representative sample of 13 of such studies are described in Table 1 that also serves to show the great range of methods used to estimate viability but all used viability at the end to estimate viability at the start of the trial. Thus, estimated initial viability as a fraction is given by: (viable seeds present at end of trial + germinants)/(total seeds in trial). The results are then corrected for viability: (seeds germinated)/(total seeds in trial × initial viability), as described by Gosling (2003).

This standard procedure economizes on the number of seeds needed for the trial as it is not necessary to 'waste' seeds by testing for viability on separate samples before the trial begins. This can be important when seeds are scarce if the species is rare, or seeds are difficult to collect or expensive

to purchase. It also removes any time-lapse loss of viability between estimating viability before the trial begins and undertaking the trial itself. Further, it expedites the testing task as only ungerminated seeds need be examined for their viability. It also avoids the need to use a mean value obtained pre-trial to apply to all treatments that ignores sample effects on the viability of seeds used as 'actual' initial viability of the seeds used in each replicate can be determined.

During a study of the germination requirements of *Leucadendron* species in relation to alternating temperatures, smoke and heat (Newton et al. 2021), I noticed that estimated initial viability using this standard procedure varied greatly between some treatments in a number species when they should have been at least non-significantly different. Estimated viability declined the lower the level of germination, i.e., usually the controls. This indicated that there might be an unanticipated treatment effect on the viability of ungerminated seeds at the end of the trial.

Our solution at the time was to abandon this method used routinely in estimating viability of species at the Millennium Seed Bank (inspection of the condition of the embryo by the 'cut' test when seeds are tested for their ability to remain viable during cold storage, Hall et al. 2017). Instead, those seeds that had not experienced microbial infection during the trial were treated as the ones initially viable. This solution proved to be unaffected by the treatment, i.e., infected seeds had indeed been randomly allocated to the various treatments. However, it is possible that some of the remaining seeds were still nonviable, although only the cut test applied at the *start* of the trial would have addressed that. In fact, the large number of species with 100% viability by the 'uninfected' method implies that initial viability may indeed have been overestimated.

I checked the literature and found that Hay and Probert (2013) had warned that seeds kept for prolonged periods under (apparently suboptimal) experimental conditions can die although they did not provide any supporting data or references. Inspection of the 13 representative papers that used the end-of-trial approach in Table 1 revealed several with variable post-trial estimates of initial viability that were not commented on by the authors. That is, estimates of initial viability varied markedly between treatments and, unexpectedly, were especially low among the untreated controls. This included Hall et al. (2017), working on species in the South African heathlands, and Gómez-González et al. (2017), working on shrub species in Central Chile.

Clarke et al. (2000) might also be considered as another example, as this would explain why they chose to use the treatment result that gave the highest estimate of initial viability. For this begs the question: why was there a difference in estimated initial viability between treatments that required a choice to be made? Was it just random error (in which case the correct solution would be to take the mean) or was it systemic? Of additional concern is that nine of the papers either did not report estimates of initial viability on a p treatment basis (so I could not assess whether this was a problem or not) or did not adjust for it in determining germination success.

As an example of the potential problem, my collation of data from Gómez-González et al. (2017) highlights a case where estimates of viability for three species were anomalously low among the controls compared with the treatments (Fig. 1). Here, the last of the four categories into which I allocated each of the 12 species showed that estimated initial viability of the control was on average 36% less than the heat treatment (100°C for 3 min). This must be an artefact of the experimental method as, at best,

viability of both should be the same (as in categories 1 and 2) or, at worst, the treated seeds would have lower viability at the end of the trial than the controls if the heat was excessive, as in category 2 (not the reverse, as here). Note that the unexpected effect occurs among species with little germination of the controls (consistent with the observations for *Leucadendron* above) but it is not unique in that respect (for example, see category 3 that also has low % germination but without reduction in viability). Gómez-González et al. (2017) did not correct their data for initial viability so that this treatment artefact was neither recognized (certainly not noted) nor used in determining germination success of the various treatments.

Based on the above findings, I have prepared Fig. 2 to show the type of pattern that can emerge when there is a treatment effect on seed viability. Here, the lower final germination, the greater the probability of viability loss during the trial. Thus, values for initial viability become the dependent variable. If this problem was not recognized, and one of the treatments (or the mean of all treatments) was used to determine initial viability then this value clearly underestimates 'true' initial viability.

Thus, end-of-trial assessment of initial viability can lead to misleading estimates of germination success if it is based on (apparent) initial viability per treatment where this varies markedly between treatments. The reason is that the treatment itself, especially the control, may unexpectedly cause some seeds to die. Taking the approach of using germination as the independent variable and viability as the dependent variable, it is evident that, the lower the germination rate, the more likely ungerminated seeds will lose viability during the trial (Fig. 2). Thus, it seems that the longer seeds sit in the medium ungerminated, the more likely they will lose viability. That is, the control treatments, where there is little germination response, are most likely to lead to viability loss. A procedural problem is therefore indicated when the controls show lower estimated initial viability than some treatments (e.g., smoke treatment among some soil-stored species in Hall et al. 2017, heat treatment of legume seeds in Gomez-Gonzalez et al. 2017 – see Fig. 1).

Possible solutions

Since seed viability loss has a time-dependent component (Ellis and Roberts 1980), if independent assessment of initial viability during the trial is not practicable, then there is a case for avoiding prolonged duration of ungerminated seeds in the treatments. But the answer is not to terminate the trial early, as species vary greatly in their rates of germination; use some other rule, such as terminate if no further germination at twice the interval since the last germination was recorded. Note that terminating the trial at different times between treatments is not a problem as the objective is for all treatments to reach the same stage (start of asymptote) rather than choosing an arbitrary common time when this stage may not have been reached among some slow-germinating treatments.

In addition, the best-fit line can be extrapolated back to the diagonal (X = Y, Fig. 2) and this value (estimated Y) used for initial viability in calculating germination success. For example, if a linear fit is used, then $Y = a/(1 - b)$ where a is a constant and b is the slope. For a power function fit, $\log Y = \log a/(1 - b)$. Note that this point may be close to 100% independent of treatment effects on viability. Probability terms (e.g., confidence intervals) can also be added to the means. If the figure is substantially < 100%, whether there is a negligible effect of treatment on the level of estimated viability or not, then either a) this represents low viability at the

start, or b) the general experimental design has led to a loss of viability. If a wide range of related species is used and most values approach 100%, the latter possibility seems unlikely. Where no trend line can be detected, other approaches are required. As one less satisfactory compromise, the treatment that gives the highest viability estimate can be used and applied it to all treatments and control (Clarke et al. 2000) that is often near the extrapolated viability value anyway (Fig. 2). At least the problem is not ignored as currently.

Conclusions

As a result of this short review, I caution against the routine use of end-of-trial assessment of initial viability in determining germination success of various treatments. Values may prove to have been affected by the experimental design. This could be particularly serious where a wide range of treatments may have vastly different effects on estimated viability. The problem may be especially difficult to detect when in fact some treatments, e.g., application of fire-type heat, can be expected to cause loss of viability.

The preference is for viability estimates to be undertaken on a separate sample of seeds just before the experimental trial begins, or concurrently with it. This is especially important where pretreatments, e.g, high temperatures, are expected to kill some seeds (Liyanage and Ooi 2017). This means that, to minimize sample effects on pretrial estimates of initial viability, the number of seeds tested needs to be at least equivalent to the number used in the various treatments. Of course, identifying empty (no embryo), damaged or infested seeds at all stages of the trial is required (Leonard, West and Ojeda 2018), as a separate task from pretrial determination of initial viability of intact seeds.

Note that there may be merit in determining the most suitable method for estimating viability before the experiment begins as a separate issue. Thus, Lamont and van Leeuwen (1988) showed that there was no difference in estimates of viability of *Banksia tricuspis* using the cut and tetrazolium tests and thus opted for routine testing with the simpler cut test. It has not been my purpose here to compare different methods of determining seed viability (of which there are many, see Table 1) but I note that sometimes results are based on germinable (imbibed) seeds rather than checking directly for viability (Herranz, Ferrandis and Martínez-Sánchez 1999). However, even imbibed seeds may be nonviable (dead tissues can imbibe), a problem that is exacerbated when there are treatment effects on viability as described here.

It is an interesting final point to consider whether end-of-trial viability should also be estimated to determine if there is an experimental design effect (Liyanage and Ooi 2017). This is important as the ability of the treatment to break dormancy is usually gauged via the level of germination. If final viability is low and treatment independent, it raises the issue whether the general germination conditions imposed are unsuitable for that species, and that other design approaches should be considered. Possibilities include using different germination media, such as washed sand; different incubation temperature regimes; different light and dark exposures, and methods that avoid anoxia due to waterlogging. The method of determining viability may also underestimate levels, e.g., meristematic tissues need to be active for responses to the tetrazolium test (Gosling 2003).

Acknowledgments: I thank the co-authors of Newton et al. (2021) for early co-operation that alerted me to the problem. The original work on *Leucadendron* (Newton et al. 2021) was supported by the Australian Research Council and the Bentham-Moxon Trust, Royal Botanic Gardens, Kew. Rosemary Newton and Philip Groom gave extra support. Otherwise I take full responsibility for the idea and execution of the manuscript, table and figures. I declare no conflict of interest.

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References

Clarke, PJ, Davison EA, Fulloon L. 2000. Germination and dormancy of grassy woodland and forest species: effects of smoke, heat, darkness and cold. *Australian Journal of Botany* 48: 697-700.

Collette JC, Ooi MKJ. 2017. Germination ecology of the endangered species *Asterolasia buxifolia* (Rutaceae): smoke response depends on season and light. *Australian Journal of Botany* 65: 283-291.

Crosti R, Ladd PG, Dixon KW, Piotto B. 2006. Post-fire germination: The effect of smoke on seeds of selected species from the central Mediterranean basin. *Forest Ecology and Management* 221: 306-312.

Daibes LF, Pausas JG, Bonani N, Nunes J, Silveira FAO, Fidelis A. 2019. Fire and legume germination in a tropical savanna: ecological and historical factors. *Annals of Botany* 123: 1219-1229.

Dayamba SD, Tigabu M, Sawadogo L, Oden PC 2008. Seed germination of herbaceous and woody species of the Sudanian savanna-woodland in response to heat shock and smoke. *Forest Ecology and Management* 256: 462-470.

Ellis RH, Roberts EH. 1980. Improved equations for the prediction of seed longevity. *Annals of Botany* 45: 13-30.

Fernandes AF, Oki Y, Fernandes GW, Moreira B. 2020. The effect of fire on the seed germination of campo rupestre species in the South American Cerrado. *Plant Ecology* 222: 45-55.

Fidelis A, Daibes LF, Martins AR. 2016. To resist or to germinate? The effect of fire on legume seeds in Brazilian subtropical grasslands. *Acta Botanica Brasilica* 30: 147-151.

Gómez-González S, Paula S, Cavieres LA, Pausas JG. 2017. Postfire responses of the woody flora of Central Chile: insights from a germination experiment. PLOS ONE 12: e0180661 https://doi.org/10.1371/journal.pone.0180661

Gosling PG. 2003.Chapter 24: Viability testing. In *Seed Conservation: Turning Science into Practice*. Pritchard, H.W., Probert, R.J., Eds.; The Royal Botanic Gardens, Kew, UK. pp. 445-481.

Hall SA, Newton RJ, Holmes PM, Gaertner M, Esler KJ 2017. Heat and smoke pre-treatment of seeds to improve restoration of an endangered Mediterranean climate vegetation type. *Austral Ecology* 42: 354-366.

Hay FR, Probert RJ. 2013. Advances in seed conservation of wild plant species: a review of recent research. *Conservation Physiology* 1(1). cot030; doi:10.1093/conphys/cot030.

Herranz JM, Ferrandis P, Martínez-Sánchez JJ. 1999. Influence of heat on seed germination of nine woody Cistaceae species. *International Journal of Wildland Fire* 9: 173-182.

Hodges JA, Price JN, Nimmo DG, Guja LK. 2019. Evidence for direct effects of fire-cues on germination of some perennial forbs common in grassy ecosystems. *Austral Ecology* 44: 1271-1284.

Jeffrey DJ, Holmes PM, Rebelo AG. 1988. Effects of dry heat on seed germination in selected indigenous and alien legume species in South Africa. *South African Journal of Botany* 54: 28-34.

Lamont BB, van Leeuwen SJ. 1988. Seed production and mortality in a rare *Banksia* species. *Journal of Applied Ecology* 25: 551-559.

Leonard, J, West AG, Ojeda F. 2018. Differences in germination response to smoke and temperature cues in 'pyrophyte'and 'pyrofuge' forms of *Erica coccinea* (Ericaceae). *International Journal of Wildland Fire* 27: 562-568.

Liyanage GS, Offord CA, Sommerville KD. 2020. Techniques for breaking seed dormancy of rainforest species from genus *Acronychia*. *Seed Science and Technology* 48: 159-165.

Liyanage GS, Ooi MK 2017. Seed size-mediated dormancy thresholds: a case for the selective pressure of fire on physically dormant species. *Biological Journal of the Linnean Society* 123: 135-143.

Newton RJ, Mackenzie BDE, Lamont BB, Gomez-Barreiro P, Cowling RM, He T. 2021. Fire-mediated germination syndromes in *Leucadendron* (Proteaceae) and their ecological correlates. *Oecologia* 196: 589–604. doi.org/10.1007/s00442-021-04947-2

Overbeck GE, Müller SC, Pillar VD, Pfadenhauer J 2006. No heat-stimulated germination found in herbaceous species from burned subtropical grassland. *Plant Ecology* 184: 237-243.

Shayanfar A, Ghaderi-Far F, Behmaram, R, Soltani A, Sadeghipour HR. 2020. Impacts of fire cues on germination of *Brassica napus* L. seeds with high and low secondary dormancy. *Plant Biology* 22: 647-654.

Table 1. Examples of studies that used assessment of seed viability and germination at the end of the experiment to estimate initial viability.

Species tested	Method of determining initial viability from end-of-experiment data	Experimental conditions	Differing initial viability between treatments	Reference
21 woody native species of Chilean matorral	Viability calculated as: those germinated during the monitoring period plus non-germinated seeds identified as viable by tetrazolium test	Moist absorbent paper in petri dishes at 20/10°C with 12/12 h light/dark for 36 d	Yes (3 species), but data not adjusted for viability	Gómez-González et al. (2017)
13 native and one introduced species (*Acacia saligna*) of South African fynbos	Viability determined as the sum of germinated seeds and seeds appearing fresh on dissection of ungerminated seeds	1% water agar at 10/20°C with a 12 h light and dark cycle for 91 d	Yes (at least 2 species)	Hall et al. (2017)
65 species commonly occurring on New England tableland (NSW Australia)	Seeds that did not germinate, but looked viable, were analysed using tetrazolium test. Viability based on treatment with highest germination plus any seeds that remained dormant but viable	Moist pad in dish at 25/15°C with a 12/12 h light/dark for 28 or 56 d	Probably (as used highest viability levels between treatments)	Clarke et al. (2000)
Asterolasia buxifolia, riparian habitat of SE Australia	Embryo dissected from 20 seeds that did not germinate and viability confirmed if embryo and endosperm intact	Moist filter paper in petri dishes at 11/3°C with 12/12 h in light/dark for 77 d	Data not adjusted for viability*	Collette and Ooi (2017)
10 native species of Mediterranean Basin maquis	Embryo dissected from 20 seeds that did not germinate and viability confirmed if embryo intact	Soil in trays in glass house for 84 d over winter-spring, Perth, Australia	Data not adjusted for viability	Crosti et al. (2006)
46 legumes species of tropical savanna, Brazil	Initial viability equals the sum of germinated and dormant seeds in control	Moist filter paper in petri dishes at 27°C with 12/12 h light/dark for 28 d	No data given at treatment level	Daibes et al. (2019)
13 species of West African savanna woodland	Cut test – condition of embryo, conducted on ungerminated seeds post-trial	Moist filter paper in bell jars at 25°C light for 30 d	No data given at treatment level	Dayamba et al. (2008)
9 species in Brazilian Cerrado	Cut test for post-treatment seed viability	0.9% water agar in petri dishes at 25°C with a	No	Fernandes et

		12/12 h light/dark for 30 d		al. (2020)
7 native perennial forb species of grasslands and woodlands of SE Australia	Cut test - condition of embryo on ungerminated seeds post-trial	1% water agar at 25/15°C with a 12/12 h light/dark for 56 d	No data given at treatment level	Hodges et al. (2019)
2 alien and 2 indigenous legume species in S African fynbos	Germination level of scarified seeds conducted at same time as other treatments	0.02% benomyl solution in petri dishes at 20°C with 12/12 h light/dark for 30 or 60 d	No data given at treatment level	Jeffery et al. (1988)
3 species of *Acronychia* in E Australian rainforest	Ungerminated seeds checked for firmness by pressing seed with forceps. Then firm seeds checked for viability via cut test	0.8% water agar in petri dishes at 25/10°C with a 12/12 h light/dark for 28 d	No data given at treatment level	Liyange et al. (2020)
9 herbaceous species in Brazilian grassland	Tetrazolium test on ungerminated seeds	Moist filter paper in petri dishes at 20°C or 25°C at 16/8 h in light/dark for 21 d	No data given at treatment level	Overbeck et al. (2005)
Brassica napus	Cotyledon condition of ungerminated seeds post-trial. Necrotic cotyledons = nonviable, yellow-milky cotyledons = viable	Moist filter paper in petri dishes at 20°C in dark for 35 d	No	Shayanfar et al. (2020)

*Viability 80% at 100°C but only 65% at the lower temperature of 80°C appears anomalous and might indicate an unexpected treatment effect on viability (they should have been the same or the reverse if there was a heat effect on viability) but no statistical analyses were undertaken.
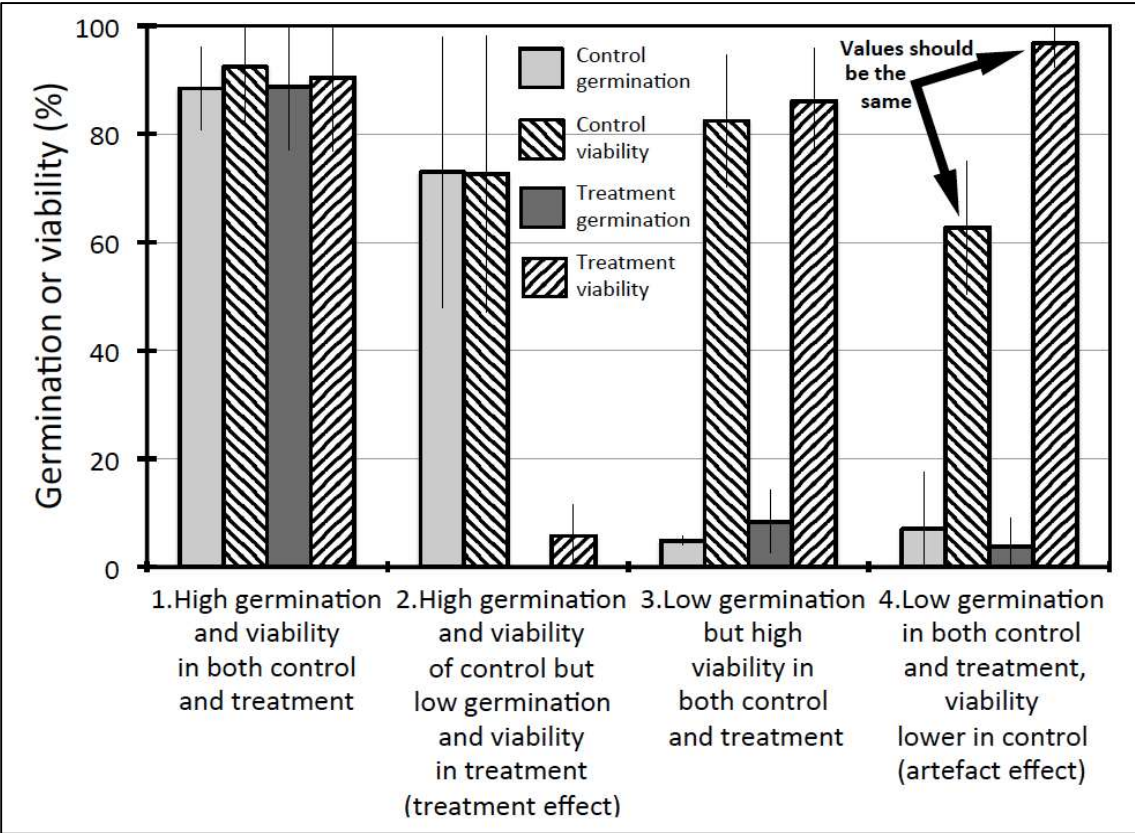
Figure 1. Example of anomalous reduction in estimated viability among some controls. I divided the data of Gómez-González et al. (2017) into four categories based on varying germination and viability pairings determined at the end of the trial with three species in each category. Results are means ± 95% CIs. Note the last category where viability of the control is on average 36% less than the heat treatment (100°C for 3 min) that must be an artefact of the experimental method. Also note that the unexpectedly low control values occur among species with little germination among the controls but it is not unique in that respect (for example, see category 3).
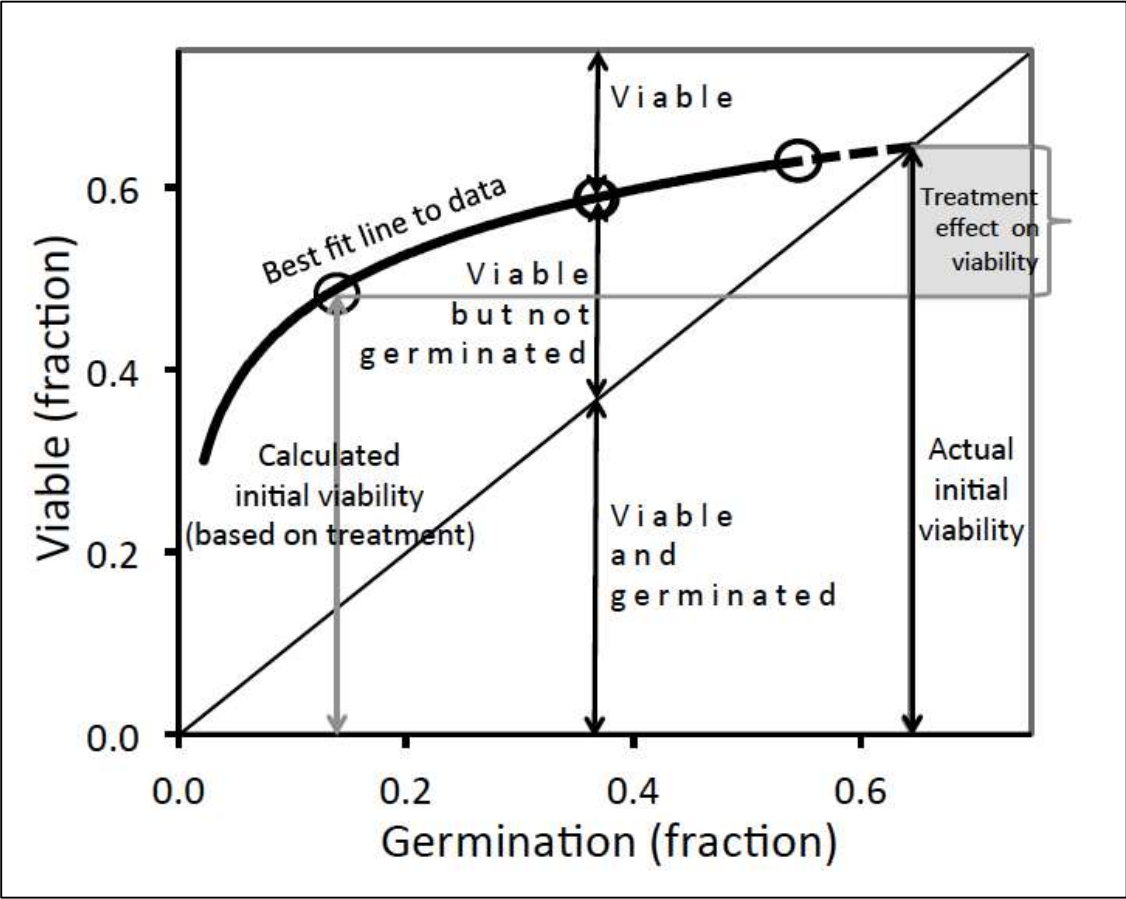
Figure 2. A hypothetical scenario where the viability of the seeds that remain ungerminated under various treatments, especially the control, lost viability during the trial (based on unpublished data of Newton et al. 2021). The circles correspond to (idealized) data points that fit on the best-fit curve. The diagonal represents the situation where all seeds that were viable, germinated. The curve can be extrapolated back to the diagonal to provide an estimate of 'true' initial viability, i.e., viability at the start of the experiment as required for estimating the success of the various treatments in breaking dormancy. The grey area represents the extent of underestimation of true initial viability if, for example, it was based on the far left data point.